Mixed lipopeptide micelles for inducing an immune response and their therapeutic uses

Background Of The Invention

The present invention relates to mixed lipopeptide micelles for inducing an immune response.

A further object is the use of these micelles for therapeutic purposes.

There are two types of effector immune responses: the humoral response due to antibodies, and the cytotoxic response due to $CD8^+$ T lymphocytes.

An effective cytotoxic response requires the presentation of the antigens to the cytotoxic $CD8^+$ T lymphocytes (CTL), in combination with class I molecules of the Major Histocompatibility Complex (MHC), but also to helper $CD4^+$ T lymphocytes (HTL) in combination with class II MHC molecules.

The use of lipopeptides for inducing a cytotoxic response, in other words the in vivo generation of cytotoxic T lymphocytes, has already been described. In particular, application FR-90 15 870 published under the n° 2 670 787 (Institut Pasteur de Lille, Institut Pasteur, INSERM) discloses lipopeptides composed of a peptide portion comprising 10 to 40 amino acids and a lipid portion which may be derived from fatty acids or steroid groups.

These lipopeptides show a good aptitude for inducing a cytotoxic response. However it was advisable to make them able to induce a better quality response by addition of a helper T response whose importance for effective induction and maintenance of the cytotoxic response is known. It was also advisable to make them able to induce a response in as many individuals as possible.

BOURGAULT et al. (1994, J. Immunol., 2530-2537) induced a CTL and HTL response from a mixture of lipopeptides, in the form of an emulsion with an oily adjuvant.

Nevertheless, it was necessary to add incomplete Freund's adjuvant (IFA). The immunogenicity of the vaccine preparation used necessarily involved the functional co-presentation of the HTL and CTL units located in one or more lipopeptides in the mixture. However, the effectiveness of the co-

presentation of the different units involved depended on the combination with the incomplete Freund's adjuvant within a very fine emulsion.

An article under the name of VITIELLO et al. (1995, J. Clin. Invest., 95, 341-349) raised the possibility of inducing a CTL response in a selected human population (HLA-A2) by using a lipopeptide containing a sequential combination of a CTL HLA-A2 antigenic determinant and a multivalent helper (HTL) antigenic determinant. It should be noted that this study was carried out on a genetically restricted population.

This article also reports an experiment during which two types of associations between the HTL antigenic determinant and the CTL antigenic determinant were compared: on the one hand, a covalent sequential combination within the same lipopeptide, and on the other an association by simple mixture of a lipopeptide containing the CTL unit with a peptide containing the HTL unit. The results of this study showed a very clear advantage of the covalent combination compared to the mixture, as performed by the authors, in other words by mixture of solutions containing DMSO and PBS buffer (the peptides or lipopeptides were kept in stock solutions at a concentration of 10 - 20 mg/ml and diluted with PBS just before use).

However, the combination within the same lipopeptide molecule of the cytotoxic and helper antigenic determinants, although able to induce an effective immune response, required the synthesis of long amino acid sequences presenting the multiple antigenic determinants able to combine with several HLA or superfamilies of class I and class II HLA. The covalent combination of all these units within a single molecule presented technical problems not easily overcome, both from the points of view of synthetic methods and analytical characterisation.

In any case, this article mentions the combination of a lipopeptide and a peptide, and not of two lipopeptides. For this reason no mixed micelle formation could take place.

Another article, published by DON DIAMOND et al. (1997, Blood, 90, n° 5), mentions the immunogenicity of a mixture between a peptide carrying a minimal CTL antigenic determinant (pp 65, sequence 495-503 of the

cytomegalovirus matrix protein) and a dipalmitoyl peptide containing an HTL antigenic determinant. The mixture was achieved by mixing solutions in dilute acetic acid or in DMSO, using an ultrasonic treatment for 15 to 30 seconds.

This article thus does not describe a mixture of lipopeptides independently containing a CTL antigenic determinant and an HTL antigenic determinant, but the mixture of a lipopeptide containing an HTL antigenic determinant and a nonapeptide corresponding to a minimal CTL antigenic determinant. In addition, there is no mention of the formation of mixed micelles or of micro-aggregates. In this particular case, however, the possibility of direct combination between the nonapeptide and the class I MHC expressed at the surface of the cells could explain the success of the approach followed. The immunogenicity of the preparation indicates that there was effectively co-presentation of the HTL and CTL antigenic determinants by the same antigen-presenting cell; however, the minimal nonapeptide used has the capacity to link directly with the class I MHC at the surface of the antigen-presenting cell, without its presentation by the cell being necessary.

The authors conclude by recognising that there are still several obstacles to long-term immunity, which confirms the experimental character of this study.

The difficulty of obtaining an immune response depending on a double presentation of the peptides separately presenting the HTL and CTL antigenic determinants is now explained by a publication by STUHLER (1997, Proc. Natl. Acad. Sci. USA, 94, 622-627). To be able to observe the induction of a CTL response, it is absolutely necessary that the HTL and CTL antigenic determinants are present on the surface of the same antigen-presenting cell (APC) to be able to be recognised at the same time by the helper T cells recognising the HTL antigenic determinant and the cytotoxic T cells recognising the CTL antigenic determinant.

It follows from the above that compositions containing within the same micelles, or the same micro-aggregates, on the one hand lipopeptides presenting a CTL antigenic determinant and on the other lipopeptides

containing an auxiliary T antigenic determinant, i.e. mixed micelles or micro-aggregates, have never, to the knowledge of the applicant, been described.

However, as described above, it is absolutely necessary that the two antigenic determinants, cytotoxic and helper T, are present on the surface of the same antigen-presenting cell.

In addition to the necessity of a co-presentation of the two antigenic determinants on the surface of the same cell, it is also essential to solubilize the lipopeptides, so as to allow their administration to patients, and their sterilisation by filtration.

Summary of the Invention

B ^ The applicant has thus endeavoured to find a solution to these different problems.

He has shown that, in order to obtain micelles individually formed from all the peptides present in the mixture, whether containing HTL or CTL antigenic determinants, it was necessary to combine the different lipopeptides after having previously dispersed them at the molecular level in a suitable solvent.

The object of the present invention is thus micelles or micro-aggregates for inducing an immune response containing at least :

- a first lipopeptide comprising at least one CTL antigenic determinant, or cytotoxic antigenic determinant, and at least one lipid unit, and

- a second lipopeptide comprising at least one helper antigenic determinant and at least one lipid unit, which may be of a different type from the first lipopeptide unit.

Brief Description of the Drawings

B ^ In the scope of the present invention, the expression "immune response" means the whole of the induced immune response, which includes the cytotoxic response and the humoral response.

The micelles according to the present invention are not limited to two lipopeptides, but may contain other lipopeptides independently presenting HTL or CTL antigenic determinants.

To understand the present invention, helper T antigenic determinant should be understood as meaning an amino acid sequence able to bind with at

least one class II HLA receptor, and able to be recognised by helper T lymphocytes.

CTL antigenic determinant should be understood as meaning an amino acid sequence able to bind with at least one class I HLA receptor and able to be recognised by cytotoxic T lymphocytes.

The helper T antigenic determinants able to bind with several different class II HLA receptors are called multivalent helper antigenic determinants (multivalent HTL).

In addition, by micelles or micro-aggregates should be understood aggregates of lipopeptides with a size making them able to be assimilated simultaneously by any antigen-presenting cell (APC) and preferably with a size less than about 1 µm.

The mixed micelles according to the invention, in other words comprising lipopeptides containing cytotoxic antigenic determinants and lipopeptides containing helper T antigenic determinants, have the advantage of combining, within the same microvolume which can be assimilated by a single APC, a wide variety of CTL and HTL antigenic determinants, without their covalent combination being necessary, while respecting the required criterion of chemical definition. Micelles which each contain a single type of lipopeptide, containing a CTL antigenic determinant or an HTL antigenic determinant, do not result in an effective co-presentation corresponding to the induction of a strong effector response.

In addition, obtaining a CTL response by the use of mixed micro-aggregates or micelles avoids the use of emulsions with oily adjuvants, such as incomplete Freund's adjuvant, whose use is not approved in human therapeutics. The micelles and micro-aggregates according to the present invention are however compatible with the use of emulsions with clinically acceptable oily vehicles.

A further advantage of the mixed micro-aggregates or micelles according to the present invention, each containing at least two types of lipopeptides, is that the solubilization of lipopeptides with a low solubility in water or in clinically

acceptable solvents, or insoluble lipopeptides, may be improved by their combination with other lipopeptide(s) with better solubility.

The micelles according to the present invention also show the advantage, compared to lipopeptides in which the HTL and CTL units are combined covalently and whose size is limited, such as those described by VITIELLO et al. (1995, cited above), of allowing the combination of a wide variety of units, and thus can be used for the vaccination of human or animal populations not selected on the basis of genetic restriction.

The micelles according to the present invention may contain a lipopeptide with at least a CTL antigenic determinant and another lipopeptide containing at least a helper antigenic determinant. However, such micelles may also contain several different lipopeptides containing different cytotoxic antigenic determinants and different lipopeptides with different helper antigenic determinants.

The lipid units of the lipopeptides may independently be one or more C_4-C_{18} units, and in particular one or more C_4-C_{18} chains derived from fatty acids, or fatty alcohols, optionally branched and unsaturated or derived from a steroid.

They may contain one or two C_4 to C_{18} lipid chains linked by a covalent bond to one or two amino acids of the peptide part. They may also be composed of two palmitic acid chains linked to the alpha and epsilon NH_2 groups of a lysine.

These lipid units may also be composed of, or contain, a residue of palmitic acid, 2-aminohexadecanoic acid, oleic acid, linoleic acid, linolenic acid, pimelautide, trimexautide, or a derivative of cholesterol, or any other natural lipid component of the cell membranes.

The lipopeptides constituting the mixed micelles or micro-aggregates are advantageously water-soluble in a proportion of at least 30% (by weight). These water-soluble lipopeptides have cationic surface-active properties, suitable for providing a solubilizing effect on other lipopeptides in weak acid medium.

The non-lipid part contains between 10 and 100, and preferably between 10 and 50 amino acids. The number of amino acids depends on the number of antigenic determinants constituting the non-lipid part of the lipopeptide and on their sizes, on the nature of the lipid part, and the proportions of the lipid and non-lipid parts.

The HTL and CTL antigenic determinants used are advantageously antigenic determinants able to bind with several different HLA, otherwise called multivalent or promiscuous antigenic determinants.

The HTL antigenic determinant used is preferably composed of the multivalent peptide 830-843 of the tetanus toxin.

6 QYIKANSKFIGITE (SEQ ID NO:1)

The glutamine (Q) of this sequence may optionally be acetylated.

Other multivalent HTL antigenic determinants may be the multivalent antigenic determinant of hemagglutinin (PREVOST-BLONDEL et al., 1995, J. Virol., vol. 62, n° 12, pages 8046-8055) or the PADRE antigenic determinant (ALEXANDER et al., 1994, Immunity, 1, 751).

The CTL antigenic determinant may be any antigenic determinant able to activate cytotoxic $CD8^+$ T lymphocytes.

It is preferably a CTL antigenic determinant of a protein presented by a tumour cell and in particular by a melanoma, a protein from HIV, from hepatitis B virus (HBV) or from papillomavirus, or protein p53.

It may in particular be one of the following antigenic determinants:

- antigenic determinants of protein BCR-ABL, resulting from the BCR-Abelson translocation (chronic myeloid leukemia) such as those listed in table 1.

- antigenic determinants of protein p53, such as those listed in table 2.

The antigenic determinants of protein p53 may in addition be comprised in the sequences 25-35, 63-73, 129-156, 149-156, 187-205, 187-234, 226-264, or 249-264 of this protein.

- antigenic determinants of proteins E_6 or E_7 of human papillomavirus (HPV), such as those listed in table 3.

- antigenic determinants of proteins of the HIV-1 virus such as those listed in table 4.

- antigenic determinants of melanoma or other tumours , such as those listed in tables 5, 6 and 7 and in particular antigenic determinants of the melan-A/mart-1 antigen of melanoma.

Other multivalent CTL antigenic determinants with a capacity to bind to class I HLA may be those included in the peptide 43-57 of HPV (GQAEPDRAHNIVTF) (SEQ ID NO:284) which contains HLA A2, A24,. B7 and B18 antigenic determinants.

The CTL antigenic determinants may also be those of parasite antigens, and in particular of *Plasmodium falciparum*.

The mixed lipopeptide micro-aggregates or micelles according to the present invention may be freeze-dried, then taken up into any clinically acceptable buffer to be administered to the patients to be treated, and in particular to patients to be vaccinated.

They may be administered by any administration route used in therapeutics and, as non-limiting examples, by parenteral, percutaneous, oral, or sublingual routes or by intra-pulmonary nebulizer.

A further object of the present invention is thus the use of these lipopeptides for the production of a drug or vaccine for inducing a specific immune response, and in particular, for inducing an immune response against cancers such as melanoma, HIV and HBV viruses, papillomavirus, p53 or malaria.

Another object of the present invention is a pharmaceutical composition characterized in that it contains a pharmacologically active quantity of one or more of the lipopeptides described above, in addition to pharmaceutically compatible vehicles.

The present invention also relates to a method of inducing an immune response against an antigen comprising the administration of micelles or micro-aggregates, such as those described above, to an individual for which such a response is sought.

An additional object is a method of immunization against a pathogenic agent comprising the administration of micelles or micro-aggregates such as those described above to an individual for whom such an immunization is sought. Such pathogenic agents, and antigens, may be those listed above.

The lipopeptides forming the micelles according to the present invention may be produced by any suitable method known to a person skilled in the art. They may in particular be obtained by the Boc-benzyl or Fmoc-tert-butyl methods, in particular as disclosed in the application FR-90 15 870, which patent application is incorporated herein by reference.

The introduction of the lipid chain may be achieved in the solid phase, after selective deprotection of the functional group or groups concerned, as described in the article by DEPREZ et al., (1996, Vaccine, volume 14, n° 5, 375-382). The lipid chain may be introduced onto the ε-NH_2 function of a lysine protected on the α-NH_2 function by an F-moc group. The Fmoc-lys (Palm) obtained may then be used in solid-phase synthesis to produce the lipopeptide.

The micelles and micro-aggregates according to the present invention may be obtained by dispersing each lipopeptide in a concentrated acetic acid solution at about 80% concentration, then mixing the solutions thus obtained.

~~The quality of dissolution, i.e. the effective dispersion at the molecular~~ level of each lipopeptide before the preparation of the mixture, is controlled by the two-dimensional nuclear magnetic resonance method (2DNMR). The resolution of the signal obtained during homonuclear experiments in two dimensions in a 600 MHz field confirms the complete dispersion, at the molecular level, of the lipopeptides in solution. The clarity of the mixture is not a sufficient criterion : in particular, the taking up of the lipopeptides by DMSO or a DMSO/water mixture does not lead, in most cases, to a sufficient dispersion state, which explains the ineffectiveness of the mixture studied by VITIELLO et al. (1995, cited above). Dissolution by acetic acid/water mixtures which are more dilute in acetic acid also does not lead in all cases to the preparation of a mixture of mixed micro-aggregates or micelles containing a statistical proportion of each constituent of the mixture at the microvolume level. In these two cases, even in the presence of an apparently clear mixture,

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the sterilizing filtration over a 0.22 µm membrane is either impossible, or irregular, with filtration yields which differ according to the constituents, which indicates that at the scale of a particle of this size, the representation of each constituent of the mixture has not been achieved. This micro-heterogeneity compromises the immunogenicity of the mixture, since it compromises the simultaneous capture and presentation of all the constituents by a single antigen-presenting cell (APC), in the case of CTL and HTL antigenic determinants present on separate lipopeptides.

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The present invention is illustrated, without in any way being limited, by the following examples.

Figure 1 represents the chemical structure of the resin of type KNORR-MBHA.

Figures 2 and 3 show the two-dimensional nuclear magnetic resonance (2DNMR) spectra of a single lipopeptide (lipopeptide ENV) and a mixture of lipopeptides respectively.

Figure 4 shows the helper response of eight macaques immunized with a mixture of lipopeptides.

Figures 5A to 5F show the cytotoxic response of macaque n° 109.

Figures 6A to 6D show the cytotoxic response of macaque n° 129.

Figures 7A and 7B show the cytotoxic response of macaque n° 127.

Figures 8, 9, 10 and 11 respectively show the cytotoxic responses of macaques n° 102, 105, 120 and 125.

Figures 12A, 12B and 12C respectively show the anti-N1, anti-G2 and anti-E cytotoxic activities of PBMC, $CD8^+$ and $CD4^+$ cells of the individual V4.1.

Figure 13 shows the cytolytic activity of PBMC of individual V4.5 collected twenty weeks after the beginning of immunization, stimulated in vitro with peptide N2 then tested for their CTL activity against wild vaccine (WT), or this same virus expressing a recombinant NEF protein (NEF, NEF-2, NEF-MN, NEF-A, NEF-ROD).

EXAMPLE 1 : Preparation of micelles or micro-aggregates according to the invention:

1- Description of lipopeptides used in the mixture

Name	Formula	Molecular weight
NEF 66	V G F P V T P Q V P L R P M T Y K A A V D L S H F L K E K G G L K(Pam)-NH_2	3862.77
NEF 117	T Q G Y F P D W Q N Y T P G P G V R Y P L T F G W C Y K L V P K(Pam)-NH_2	4017.754
NEF 182	E W R F D S R L A F H H V A R E L H P E Y F K N K(Pam)-NH_2	3451.04
GAG 183	D L N T M L N T V G G H Q A A M Q M L K E T I N E E A A E W D R K(Pam)-NH_2	3983.65
GAG 253	N P P I P V G E I Y K R W I I L G L N K I V R M Y S P T S I L D K(Pam)-NH_2	4063.05.
ENV	T R P N N N T R K S I H I G P G R A F Y A T G E I I G D I R Q A H K(Pam)-NH_2	4027.69

CTL antigenic determinants represented :

R P N N N T R K S I	HLA-B27	T Q G Y F P D W Q N Y	HLA-B62
P P I P V G E I Y	HLA-B35	Y F P D W Q N Y T	HLA-B17, B35
K R W I I L G L N K	HLA-B27	T P G P G V R Y P L	HLA-B7
L G L N K I V R M Y	HLA-B62	R Y P L T F G W	HLA-B27.2
Q V P L R P M T Y K	HLA-A3, A11, B27.2	Y P L T F G W C	HLA-B18
V P L R P M T Y	HLA-B35	A F H H V A R E L	HLA-B52
A V D L S H F L	HLA-B62	F L K E K G G L	HLA-B8
A V D L S H F L K	HLA-A11		

This set of antigenic determinants shown above should lead to the induction of CTL responses in a large proportion of the human population, on the condition of being able to benefit from the helper effect of HTL antigenic determinants which, although not defined, are very probably present for simple statistical reasons in any one of the lipopeptides on condition however of bringing together all the antigenic determinants, and thus all the peptide constituents of the mixture, in each micro-unit of volume.

2 - Synthesis

The solid phase approach was selected, using the Fmoc strategy for protecting the α-amine function, and t-Bu for protecting the side chains. The protocol used was a standard protocol based on the synthetic methods described by ATHERTON (Solid-phase synthesis, a practical approach, IRL

Press, 1989) and FIELDS and NOBLE (Int. J. Pept. Prot. Res., 1990, 35, 161-214).

The Fmoc-Lys(Palm)-OH was coupled to a resin of KNORR-MBHA type (figure 1). After deprotection of the alpha-amine function, the first amino acid was coupled (for example Fmoc-Leu-OH in the case of NEF 66). The coupling agent was TBTU (3 eq) in the presence of DIPEA (4.5 eq), with verification of coupling by a colorimetric test. A systematic acetylation was performed after a negative reaction had been obtained with this test, to minimize the risk of obtaining peptides by deletion. This succession of operations was repeated until all the amino acids in the sequence had been added.

After the synthesis, and deprotection of the terminal Fmoc group, the peptides were deprotected and cleaved by a TFA/water/DTT mixture (NEF 66, ENV), TFA/water/DTT/Ac-Trp-OH (GAG 183, GAG 253, NEF 117) or TFA/water/EDT/Ac-Trp-OH (NEF 182).

The peptides were each purified on a Vydac C18 column which was exclusively used for this purpose, at ambient temperature, with a water-acetonitrile solvent system, in perchlorate or TFA buffer.

They were then converted into their acetate form by ion exchange on a Dowex SBR column, then freeze-dried in 40% acetic acid.

Each peptide was produced from a single batch of synthesis and purification. No recycling of purification fractions was performed.

3 - Studies of the solubility of the lipopeptides:

3-1) Use of pure water:

The peptides NEF 66, NEF 117, NEF 182 and ENV could be dissolved in pure water, at concentrations of up to 5 mg/ml. Peptide NEF 117 however gave a slightly opalescent solution. Peptides GAG 182 and GAG 253 were not soluble under these conditions.

The mixture of lipopeptides was however soluble in pure water, indicating that the hydrophilic lipopeptides were having a solubilizing effect on the less soluble peptides.

3-1) Use of DMSO:

The dissolution of lipopeptides is often performed using aqueous solutions of DMSO (dimethyl sulfoxide). This very powerful organic solvent is in fact compatible, after dilution, with the majority of biological tests carried out on cells or animals, even humans. The use of DMSO proved effective for good solution of peptides GAG 182 and GAG 253; the solutions obtained could then be diluted with water to reach a final concentration of 1 mg/ml in 20% DMSO/water; in these conditions, most of the peptides gave a clear solution, except GAG 183 for which a suspension was obtained.

It is useful to emphasize that even in the case of the clear solutions, and despite the compatibility of DMSO with Durapore filters, the solutions of lipopeptides in DMSO could not be filtered over filters of porosity 0.22 µm, because they exerted a pressure incompatible with the mechanical resistance of the filters. This observation shows the formation of aggregates of size greater than 0.22 µm. In some cases, we found it impossible to filter over filters resistant to solvents, with porosity 1 µm, because of the formation of gels (this size of filter is in fact used to filter concentrated lipopeptide solutions before purification by RP-HPLC).

3-3) Use of 25% concentrated acetic acid:

The inclusion of the necessary step of sterilizing filtration thus requires the use of an organic solvent more suitable for dissociating the aggregates, compatible after dilution with freeze-drying, and non-toxic at low doses. Acetic acid was tested.

A minimum quantity of this solvent was initially used, defined as the quantity giving clear solutions at concentrations of 5 mg/ml : for peptides GAG 183 and GAG 253, 25% acetic acid was used; for the other peptides, dissolution in pure water was performed.

The solutions were subjected to nuclear magnetic resonance analysis in a 600 MHz field. It was observed that, despite the apparent clarity of the lipopeptide solutions of this series, even the most hydrophilic lipopeptides formed aggregates of significant size which prevented this type of study, in the absence of resolved signals.

Dissolution under these conditions did not lead to statistical dispersion, at the molecular level, of each of the constituents, despite the apparent clarity of the solutions.

3-4) Use of 80% concentrated acetic acid:

The use of 80% concentrated acetic acid was then tested. The peptides were dissolved at a concentration of 1mM in 1 ml of 80% acetic acid (corresponding to : NEF 66 : 3.86 mg/ml; NEF 117 : 4.02 mg/ml; NEF 182 : 3.45 mg/ml; GAG 183 : 3.98 mg/ml; GAG 253 : 4.063 mg/ml; ENV : 4.027 mg/ml).

Analysis of the lipopeptides by proton NMR at 600 MHz

The lipopeptide samples were prepared by dissolving the lipopeptides in a solution of acetic acid (CD3COOD, 99.5% D atoms, EURISO-TOP, France)/H2O; 80:20 (V:V). 4 µl of a 50 mM solution of TMSP [sodium 3-(trimethylsilyl)propanesulfonate] in D2O were added as chemical shift reference. The final concentration of each peptide was 1 mM in at least 2 ml of solvent, which were transferred into 8 mm diameter NMR tubes (WILMAD 513A-7PP, Interchim, France).

The proton NMR spectra were performed on a BRUKER DMX600 NMR spectrometer fitted with an 8 mm BBI probe with z gradient, at a sample temperature of 310°K.

NOESY (Nuclear-Overhauser effect spectroscopy) experiments in two homonuclear dimensions according to Kumar et al. (1980, Biochem. Biophys. Res. Comm., 95, 1-6) and TOCSY (Total Correlation Spectroscopy) according to Bax and Davis (1985, J. Magn. Reson., 65, 355-360) and Griesinger et al. (1988, J. A. C. S., 110, 7870-7872) were obtained with 2048 x 512 complex points and processed after multiplication in two dimensions by a sine wave displaced by $\Pi/4$ with 2048 x 1024 points, for a spectral window of 12 ppm. The mixture times were 300 ms for the NOESY and 160 ms for the TOCSY. During the TOCSY mixture time, a MLEV 16 was applied with a B1 field of 7.8 KHz. So as to be under the same temperature conditions, the spin-lock time of the TOCSY was applied without resonance (+ or - 1 MHz) in the NOESY. The

suppression of water was achieved by using a slight pre-saturation of this signal during the relaxation time and the mixture time of the NOESY.

The high-field NMR analysis of the solutions showed perfectly resolved signals, allowing the TOCSY-NOESY experiments, and the complete sequential attribution of each lipopeptide. This result indicates complete dispersion, at the molecular level, of the lipopeptides in 80% acetic acid.

The 2D NMR spectrum of peptide ENV is shown on figure 2. The spectra of all the peptides could be obtained under the same conditions and interpreted. In order to verify if the intermixture of the solutions would change the dispersion of the lipopeptides, a 2D NMR spectrum of a virtual mixture was obtained by superimposing the 6 spectra obtained individually onto a single representation. It was compared with the 2D NMR spectrum actually obtained by mixing the solutions (figure 3). The resolution of the signals remained comparable, proving that none of the peptides had altered the solubility of the other constituents of the mixture. The sequence analysis required an accumulation of signals over 120 hours for each lipopeptide, during which period no significant alteration of the peptides was detected, either by NMR or by RP-HPLC. This observation thus allows the use of this solvent for the solubilization of the lipopeptides, their mixture, then filtration, even with a residence time of the order of 1 to 2 hours, conceivably necessary for the handling of relatively large volumes.

4 - Studies of the sterilization filtration step:

4 - 1 Isolated lipopeptides:

Tests on the sterilizing filtration were performed on 5 mg/ml solutions of each lipopeptide in water for peptides NEF 66, NEF 117, NEF 182 and ENV and in 25% acetic acid for peptides GAG 183 and GAG 253. The filtration yields for 1 ml over Millipore Millex GV SLGV 0130S filters (0.22 µm), followed by freeze-drying, are shown in table 8 (to within the precision of the determination).

These results are in agreement with the solution studies performed by NMR, and give information on the size of the aggregates or micelles detected: some peptides form aggregates of size greater than 0.22 µm, and as a result

lead to mixtures containing micro-heterogeneities and an improbable simultaneous capture at the scale of the antigen-presenting cell.

4 - 2) Preparation of different lipopeptide mixtures:

a) Preparation of batch CK2. Simple mixture of solutions, and production of a clear but micro-heterogeneous solution:

For the dissolution of the lipopeptides and the preparation of the mixture, solutions of totally clear appearance were intermixed, so as to evaluate the possible contribution of the surface-active character of lipopeptides ENV, NEF 66, NEF 117 and NEF 182. The conditions are summarized in table 9.

The solutions obtained were subjected to ultrasonic action to encourage the dispersion of the aggregates, mixed to give a final volume of 5.5.ml, and the mixture was again subjected to ultrasonic action, then diluted with 9.5 ml of water to obtain a final concentration of about 8% in acetic acid (AcOH), compatible with a good quality freeze-drying. This solution, after a final period in the ultrasonic bath, was filtered over Millipore Millex GV SLGV 0130S filters (0.22 µm). The filtration yields for the peptides in the mixture were calculated for each lipopeptide, to give the results shown in the final column of table 9 (to within the precision of the determination).

The heterogeneity of the yields depending on the peptide showed the heterogeneity of the solution. Each peptide behaved as if it had been filtered individually : this behaviour was particularly evident for the peptide GAG 253, whose filtration yield from this solution in 8% acetic acid was lower than the yield observed when it was filtered alone from a solution of 25% acetic acid. This result confirms that, despite the apparent clarity of the mixture in dilute acetic solution, the mixture between the lipopeptides had not formed mixed micro-aggregates or micelles which contained in particular the more hydrophobic peptides. The exchanges of the lipopeptides between micelles occurs poorly under these conditions, and the surface-active function of lipopeptides ENV, NEF 66, NEF 117 and NEF 182 could not operate.

b) Preparation of batch CK3 : preparation of mixed micelles or micro-aggregates not including micro-heterogeneity

In order to guarantee complete mixing of the different lipopeptides at the level of each micro-unit of volume, a different strategy was followed :

- each lipopeptide was dissolved in 80% acetic acid so as to exploit the dissociating properties of this solvent.

- in order to exploit the cationic surface-active properties expected of the peptides ENV, NEF 66, NEF 117 and NEF 182 in weak acid medium during the dilution step, the lipopeptides were dispersed in 80% acetic acid in the following order : 1 : ENV , 2 : NEF 66 , 3 : NEF 117 , 4; NEF 182, ending with the dispersion of the two most hydrophobic lipopeptides in a solution now concentrated in dissociating agents : acetic acid and cationic detergents. The fifth lipopeptide introduced was GAG 183 and the sixth GAG 253. An ultrasonic step was used at each stage to ensure effective dispersion of the aggregates.

The solutions were mixed, then filtered over Millex GV SLGV 0130S filters (0.22 µm). The filtration required a lower pressure than during the filtration of the 8% solution. The receiver vessels and the filter were then rinsed with water, in sufficient quantity to give a final acetic acid concentration of 8% (final volume 15 ml as before), so as to ensure the quality of the freeze-drying step. The filtration yields of the peptides in the mixture were calculated for each lipopeptide, to give the results listed in the final column of table 10 (to within the precision of the determination).

The homogeneity of the yields confirms the homogeneity of the solution resulting from the dispersion at the molecular level at the time of filtration in concentrated acetic acid. The subsequent dilution cannot result in a reorganization of each peptide into monovalent entities, by application of the laws of entropy. This method of preparation of the mixture thus gives mixed micelles which each necessarily contain a statistical representation of each lipopeptide. The surface-active properties of the lipopeptides can operate and guarantee the solubility in water of vaccine doses after freeze-drying as well as the stability of the solutions during the handling time.

c) Preparation of batch CK9

The procedure used was the same as for the previous batch, apart from the quantities. The solution of the peptide (20 mg/ml in 80% acetic acid) was filtered in 4 portions, changing the filter before its saturation, using identical membranes (Durapore STERIVEX GV 0.22 µm sterile units (Millipore)), then made up with the water used for rinsing the filters and for dilution. The final volume was 1516 ml (including 154 ml of acetic acid : 10% in final solution). The portion volume was 1.3 ml per dose. The apportioned doses were freeze-dried, and analysed using a validated HPLC determination method. The filtration yield for each lipopeptide is given below in table 11, and takes into account the determination sensitivity of each lipopeptide.

During the preparation of this batch, we again observed good homogeneity of the filtration yields, confirming the formation of mixed micelles or micro-aggregates, each micro-unit containing an equivalent proportion of each constituent of the mixture.

The mixture after dilution and freeze-drying gave a white powder forming a compact homogeneous cake, which could very easily be taken up into solution in pure water or a solvent able to restore the osmolarity of the solution (5% glucose, 5% mannitol). The solution showed a very slight opalescence. The pH obtained after taking up in a non-buffered solvent was 4.90. Raising the pH by 1 unit caused a slow precipitation: this behaviour contributed to the formation of a deposit during subcutaneous or intramuscular injection.

d) Test of uniformity of concentration on batch CK9

According to the Pharmacopoeia, powders for parenteral use are subjected to a requirement of uniformity of concentration. The test must be performed on 10 random samples, which are analysed individually for the active ingredient using an appropriate analytical method. The preparation satisfies the test if the concentration of each sample is between 85 and 115% of the average concentration.

The test was performed on 15 random samples, taken up in solution and diluted in 80% acetic acid according to a standardized operational procedure, so as always to inject an identical proportion of about 15 µg, defined during

preparation of the calibration curve. Each sample was injected three times, the concentration of each active ingredient corresponding to the average of the three values obtained.

The values obtained are given in table 12 below. The distribution of the values is clearly statistical. All the values are within a range defined for a two-sided test with P = 0.975 (except for three deviant values, all from flask n° 1, which may correspond to a dilution error). The minimum and maximum values defined are within the limits imposed by the Pharmacopoeia (the difference observed was within 4 and 14.95% depending on the peptide, a variation linked to the inherent imprecision of the analysis method).

The absence of micro-heterogeneity of the solution was confirmed by the fact that the apportioned vaccine doses satisfied the concentration uniformity test.

EXAMPLE 2 - Preparation of a mixture of lipopeptides (SIV-Mortara 1) and test of immunogenicity in macaques.

1) Preparation of the batch SIV-Mortara 1

This small batch was prepared in order to perform a pre-clinical test on macaques, to verify the tolerance and immunogenicity. This batch resulted from the mixture of the following lipopeptides :

Name	Formula
NEF 101	S V R P K V P L R A M T Y K L A I D M S H F I K E K K(Pam)-NH_2
NEF 125	E K G G L E G I Y Y S A R R H R I L D M Y L E K(Pam)-NH_2
NEF 155	D W Q D Y T S G P G I R Y P K T F G W L W K L V K(Pam)-NH_2
NEF 201	S K W D D P W G E V L A W K F D P T L A Y T Y E A K(Pam)-NH_2
NEF 221	Y T Y E A Y A R Y P E E L E A S Q A G Q R K R L E E G K(Pam)-NH_2
GAG 165	K F G A E V V P G F Q A L S E G C T P Y D I N Q M L N C V G D K(Pam)-NH_2
GAG 246	Q I Q W M Y R Q Q N P I V G N I Y R R W I Q L G L Q K C V R M Y N P T N K(Pam)-NH_2
TT	Ac-Q Y I K A N S K F I G I T E L K K K(Pam)-NH_2

Their mixing was performed from solutions in concentrated acetic acid, as for the preparation of batch CK3.

2) Immunogenicity in macaques.

a) Materials and methods

The macaques, respectively numbered 102, 105, 109, 117, 120, 125, 127 and 129 were immunized by subcutaneous injection of the batch prepared above (500 µg), in sterile water, and were reinjected after periods of thirty days and sixty days.

These immunizations were performed in accordance with the directives of the European Union.

Preparation of CTL lines

Blood cells (PBMC) were isolated by density gradient centrifugation through a lymphocyte separation medium (Pharmacia, Uppsala, Sweden). They were used immediately, or stored at -180°C in liquid nitrogen. Anti-peptide CTL lines were obtained by cultivating the monkey PBMC (2 x 10^6 cells/ml) in microtitration plates, in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), non-essential amino acids (1%), sodium pyruvate (1 mM), HEPES buffer (10 mM), 2-mercaptoethanol (2 x 10^{-5} M) and 10% fetal calf serum (FCS) inactivated by heat.

The mixture of the seven free peptides, in other words without the lipid unit (5 µM of each), corresponding to the lipopeptide sequences was added to each well.

The plates were then incubated for three days at 37°C and interleukin-2 was added to each plate (10 IU/ml).

After seven days and fourteen days, the effector cells were stimulated by addition of new autologous PBMC, which had been in contact with the peptide mixture (5 µM of each) for two hours, then washed and irradiated (4000 rads).

Determination of the proliferation of T cells

PBMC cells (2 x 10^5 in 200 µl per well) were cultivated in plates containing 1 µg/ml of lipopeptide TT (830-846), and 10 µg/ml of the peptide from tetanus toxin (TT).

After five days of culture, 1 µCi of [^{3}H]TdR was added to each well and the incubation was continued for eighteen hours. The cells were then collected

using an automatic cell collector then the incorporation of tritiated thymidine was quantified using a scintillation counter.

Phenotypic analysis of CTL cell lines

The phenotype of the cell lines was determined the day that the chromium release assay was performed, by incubating the cells with anti-CD4 conjugated to FITC (OKT4, Ortho Diagnostic Systems, Raritan, NJ) and with anti-CD8 conjugated to phycoerythrin (Leu-2a, Becton Dickinson, Mountain View, CA) for thirty minutes at 4°C. The cells were washed with PBS buffer, then the percentage of coloured cells was determined using an Epics Elite flow cytometer (Coulter, Margency, France). Antibodies presenting a mixture of isotypes were used as controls.

In vitro conversion of B (B-LCL) cell lines

B (B-LCL) cell lines were obtained by incubating series dilutions of PBMC using the supernatant of cell line S 594. This line is infected by baboon herpes virus which immortalizes the cells (herpes virus papio). The B-LCL were then cultivated in the culture medium supplemented with 10% FCS.

Chromium release assay

The target cells were sensitized with the peptides. 10^6 B-LCL cells were incubated either overnight or for 1 hour, respectively, with the long or short peptides (concentration range 10^{-5}M - 10^{-8}M) at 37°C in a humid atmosphere with 5% CO_2. In order to obtain the target cells presenting the products of the SIVmac gene, the B-LCL were incubated at a concentration of 10^6 cells/ml with a recombinant vaccine virus (20 PFU/cell) for eighteen hours under the same conditions. The B-LCL were then washed and marked with 100 µCi $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Courtaboeuf Les Ullis, France) for 1 hour, washed twice and used as target cells. The ^{51}Cr release was performed in microtitration plates. The cytolytic activity of the anti-SIV cell lines was measured by mixing 5 x 10^3 target cells marked with chromium with the effector cells, at various ratios of effector cells to target cells, in a final volume of 200 µl/well. The plates were incubated for 4 hours at 37°C, then 100 µl of supernatant was taken from each well and analysed in a gamma radiation counter.

The spontaneous release of chromium was determined by incubating the target cells with medium alone. It never exceeded 20% of the total chromium incorporated.

The specific release of chromium was measured as follows:

100 x (experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm).

The variation within a sample never exceeded 5%.

b) Results

Figure 4 shows the helper T response of the eight macaques.

Figures 5 to 11 show the cytotoxic response of the macaques.

The results of the immunizations with different peptides are summarized in table 13.

They show that seven of the eight macaques tested recognized different peptides, with macaques n° 109, 129 and 127 showing a particularly strong response.

The effectiveness of the induction of a CTL response confirms that the APC of the animals were able to capture and present one or more CTL antigenic determinants, and simultaneously the strong helper antigenic determinant present in the tetanus anatoxin and recognized by some of the animals.

EXAMPLE 3:

Preparation of a lipopeptide mixture (batch HG 1) for clinical tests in man.

A mixture of lipopeptides was defined for performing a clinical test (VAC 10), combining within the micelles the same peptide TT with sequences selected on the selection principle developed for VAC 04 (existence of one or more CTL antigenic determinants per sequence).

- the cysteine of peptide NEF 117 was replaced by a leucine : after synthesis and tests on cellular tests of several analogs of the CTL antigenic determinant nonapeptide containing this amino acid, it was observed that this replacement was possible; this modification avoided the stability problems due to the formation of a disulfide bridge.

- the peptide GAG 17 was selected from among other candidates for its strong cationic surface-active character, able to help to keep the other peptides in solution, and in particular GAG 253, which was retained in the mixture because of its immunogenicity in man.

The composition of this mixture, in which Pam represents a unit derived from palmitic acid and Ac the acetyl group, was the following :

Name	Formula
GAG 17	E K I R L R P G G K K K Y K L K H I V K(Pam)-NH_2
GAG 253	N P P I P V G E I Y K R W I I L G L N K I V R M Y S P T S I L D K(Pam)-NH_2
POL 325	A I F Q S S M T K I L E P F R K Q N P D I V I Y Q Y M D D L Y K(Pam)-NH_2
NEF 66	V G F P V T P Q V P L R P M T Y K A A V D L S H F L K E K G G L K(Pam)-NH_2
NEF 116	H T Q G Y F P D W Q N Y T P G P G V R Y P L T F G W L Y K L K(Pam)-NH_2
TT	Ac-Q Y I K A N S K F I G I T E L K K K(Pam)-NH_2

This set of peptides was synthesized as described in the previous examples. The mixture of solutions was performed on a sample of 5 mg of each peptide, dissolved at a concentration of 20 mg/ml in 80% acetic acid then mixed in the following order : 1 : GAG 17; 2 : NEF 66; 3 : NEF 116; 4 : TT; 5 : GAG 253; 6 : POL 325.

The yields from the operation of filtering the concentrated acetic acid solutions, followed by a dilution with water, proved comparable to the yields observed for the same operations with the mixture CK3 (to within the precision of the determination). The homogeneity of the solubilities and the behaviour during the sterilizing filtration despite the heterogeneities of their individual chemical behaviour indicated the formation of mixed micelles.

EXAMPLE 4:

Preparation of a mixture of lipopeptides derived from antigen LSA3 for pre-clinical vaccination tests against the intrahepatic stage of *Plasmodium* falciparum, performed in mice and chimpanzees, then a clinical test in man.

Name	Formula
LSA3 CT1	L L S N I E E P K E N I I D N L L N N I K(Pam)-NH_2
LSA3 NRI	Ac-D E L F N E L L N S V D V N G E V K E N I L E E S Q K(Pam)-NH_2
LSA3 NRII	Ac-L E E S Q V N D D I F N S L V K S V Q Q E Q Q H N V K(Pam)-NH_2
LSA3 RE	K(Pam) V E S V A P S V E E S V A P S V E E S V A E N V E E S V A E N V-NH_2

This set of peptides was synthesized as described in example 1. The mixture of solutions was performed on a sample of 5 mg of each peptide previously dissolved at a concentration of 20 mg/ml in 80% acetic acid then mixed in the following order : 1 : LSA3 NRI; 2 : LSA3 NRII; 3 : LSA3 CT1; 4 : LSA3 RE. The yields from the operation of filtering the concentrated acetic acid solutions, followed by a dilution with water, proved comparable for all the lipopeptides.

EXAMPLE 5:

Study of the immune response in man after injection of micelles from batch CK9.

1. Materials and methods

Micelles used:

The micelles which were injected were obtained as described in example 1 for batch CK9.

Long and short peptides.

The following long peptides corresponding to the immunogenic lipopeptides were synthesized (the positions of the amino acids on proteins NEF, GAG and ENV are given in parentheses) : N1 (NEF 66 to 97), N2 (NEF 117 to 147), N3 (NEF 182 to 205), G1 (GAG 183 to 214), G2 (GAG 253 to 284) and E (ENV 303 to 335).

The following short peptides, including the lipopeptide sequences already known to be the minimal CTL antigenic determinants, were synthesized by Neosystem (Strasbourg, France):

NEF 121-128, NEF 137-145, NEF 184-191 and NEF 195-202 restricted to HLA-A1.

NEF 136-145, NEF 190-198 and GAG 183-191 restricted to HLA-A2.

NEF 73-82, NEF 84-92 and EBNA 4 (SEQ ID NO: 285) 416-424 HLA restricted to HLA-A11.

NEF 90-97 and NEF 182-189 restricted to HLA-B8.

NEF 134-141 and GAG 263-272 (SEQ ID NO. 10) restricted to HLA-B27.

NEF 135-143 restricted to HLA-B18.

Immunization protocol:

Volunteers were immunized by subcutaneous injection of the micelles, or the six corresponding lipopeptides, in the presence of QS21 adjuvant. The lipopeptides or micelles were injected in different ways depending on the individual.

Volunteers V4.6, V4.15, V4.16, V4.17, V4.18, and V4.28 were immunized with the six lipopeptides in the form of micelles.

Volunteer V4.6 received 250 µg of each of the lipopeptides.

Volunteers V4.15, 4.16, V4.17, V4.18, and V4.28 were immunized with 500 µg of each of the six lipopeptides.

Volunteers V4.5, V4.1, V4.19, V4.21, V4.32 and V4.34 were immunized with the six lipopeptides in the presence of QS21 adjuvant.

Volunteer V4.5 received 100 µg of the 6 lipopeptides, while volunteers V4.1, V4.19, V4.21, V4.32 and V4.34 each received 500 µg of the six lipopeptides.

All the volunteers were immunized three times with the mixture of the six lipopeptides, the two later injections being performed 4 weeks and 16 weeks respectively after the first injection.

Blood samples were taken after the first injection (hereafter referred to as week 0) and 20 weeks after the first injection (week 20).

Peripheral blood mononuclear cells (PBMC) and serum were isolated by conventional methods, and frozen.

ELISA detection of HIV anti-peptide antibodies of immunoglobulin G (IgG) type.

Wells of polystyrene plates were covered with 5 µg/ml of the peptides (N1, N2, N3, G1, G2 or E) overnight at 4°C. Saturation was performed using a PBS solution containing 0,1% Tween 20 and 3% bovine serum albumin (BSA). Diluted serums (1/100) were incubated in the covered wells overnight at 4°C and the bound antibodies were detected using goat anti-human IgG conjugated with alkaline phosphatase (1/5000, Sigma). The phosphatase activity was measured using 4-methyl umbelliferyl phosphate as substrate (Sigma), and the fluorescence measurement was performed at 360/460 nm in a Cytofluor 2300 (Millipore).

Measurement of the T cell responses directed against HIV peptides.

PBMC (10^5 per well) were cultivated in complete medium with 1 µg/ml or 0.2 µg/ml of the soluble peptides (N1, N2, N3, G1, G2 or E). The proliferation was determined after 5 days culture by added 1 µCi/well of tritiated thymidine (NEN, Paris) 12 hours before their collection.

The capacity of the PBMC to proliferate in vitro was verified using independent cultures performed over 5 days with phytohemagglutinin A (PHA) of PPD (Tuberculin purified derivative Reference Statens Serumins Institute n° 2390), tetanus toxin (TT) and SEB (enterotoxin B of Staphylococcus golden, Reference Sigma S4881), at 1 µg/ml and 10 µg/ml respectively.

Removal of the $CD4^+$ and $CD8^+$ T cells of the PBMC was performed with anti-mouse immunoglobulins and by complement activation. To summarize, 10^7 PBMC were incubated in 1 ml of medium lacking bovine serum albumin for 30 minutes at 4°C with 2 µg of monoclonal antibody OKT4 or OKT8 (Ortho Diagnostic Systems).

1 ml of diluted rabbit serum complement (Hoechst Behring, Reuil, France) was added over 45 minutes at 37°C. The cell suspension was washed twice so as to remove the unbound complement. The resuspended cells were analysed using flow cytometry. Analysis of the phenotypes using anti-$CD4^+$ and anti-$CD8^+$ antibodies was performed to verify the enrichment. Finally, the

cells resulting from the removal of the $CD4^+$ and $CD8^+$ cells were tested in a proliferation test.

Preparation of CTL cell lines

In vitro stimulation of the PBMC was performed by mixing 10^6 PBMC (responsive cells) with 10^6 irradiated stimulant cells (autologous PBMC incubated for 2 hours with different peptides) in complete RPMI culture medium (RPMI 1640 supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, nonessential amino acids and 10% heat-inactivated bovine serum albumin).

10 U/ml of interleukin -2 were added after 3 days. The responsive cells were restimulated each week for 3 or 4 weeks using peptides incubated with autologous PBMC (prepared in the same way as on day 0), in a medium supplemented with 10 U/ml of interleukin-2. After 3 or 4 stimulations, the CTL cells were tested using the EBV autologous cell line as target overnight with 10 µg of the different peptides (N1, N2, N3, G1, G2 or E) for 10^6 cells.

In order to obtain the target cells presenting the products of the HIV gene, EBV target cells were infected, at a rate of 10^6 cells/ml, with a wild type (WT) vaccine virus or with HIV-1/LAI, HIV-1/MN, HIV/A or HIV/ROD NEF recombinant vaccine viruses overnight (20 PFU/cell).

The different target cells were then washed and marked with 100 µCi of $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Les Ullis, France).

The cytolytic activity was measured in a ^{51}Cr release test, over 4 hours. The average spontaneous release did not exceed 20% of the total ^{51}Cr incorporation.

The results are expressed as follows :

specific release of chromium = 100 x (measured cpm/spontaneous cpm)/(maximum cpm - spontaneous cpm).

The removal of the $CD4^+$ and $CD8^+$ cells from the PBMC was performed as described above.

ELISPOT γ-interferon test

96-Well microcells plates (MultiScreen-HA, Millipore S.A., Molsheim, France) were covered with 5 µg/ml of mouse anti-human-γ-interferon antibody,

as capture antibody (Genzyme Corporation, Cambridge, Massachusetts, USA) overnight at 4°C.

After washing, the wells were saturated with complete RMPI medium and PBMC which had been freshly isolated, or kept cold, were added (2 x 10^5 cells per well) with different peptides corresponding to the minimal $CD8^+$ antigenic determinants (10 µg/ml).

After 24 hours incubation at 37°C in an incubator (5% CO_2), the plates were washed and incubated for 2 hours with 100 µl rabbit anti-human-γ-interferon polyclonal antibody (1/250, Genzyme). After washing, a rabbit anti-IgG-biotin conjugate (1/500, Boehringer Mannheim France S.A., Meylan, France) was incubated for 1 hour. Finally, extravidine marked with alkaline phosphatase (Sigma-Aldrich Chimie S.A.R.L., St Quentin Fallavier, France) was added over 1 hour.

100 µl of alkaline phosphatase chromogenic substrate (Bio Rad Laboratories, Hercules, CA, USA) were added to develop the spots. The blue spots were then counted using a microscope.

The negative control consisted of PBMC incubated alone in the medium, or incubated with a peptide corresponding to a $CD8^+$ antigenic determinant derived from the HIV virus presented by adapted HLA.

The positive control consisted of activating the PBMC with 50 mg/ml of PMA (Phorbol myristate acetate, reference Sigma P 8139) and 500 ng/ml of ionomycin (100 to 300 PBMC per well were added).

This strong mitogen stimulation allowed measurement of the viability of the T lymphocytes, and verification of the quality of the storage in the cold.

2. Results

Tolerance of the treatment.

The secondary effects from the injection of the lipopeptides were not serious. An epidermal reaction was observed at the injection site. Local reactions consisted of small erythemas lasting only 24 to 48 hours. These effects were in no case associated with systemic symptoms. These observations show that the lipopeptides are well tolerated in normal individuals.

Specific induction of a humoral response against HIV-1 peptides

Serum samples were collected before the start of the vaccinations (week 0) and at the twentieth week, after the third injection.

The serums from the immunized volunteers were tested by ELISA for the presence of IgG antibody directed against the NEF (N1, N2, N3), GAG (G1, G2), and ENV (E) peptides

No IgG specific for the HIV peptides was detected before the injection in the twelve subjects listed in table 14.

At the twentieth week, anti-N1 IgG antibodies were detected in five of the vaccinated subjects (V4.6, V4.28, V4.1 (SQ21), V4.32 (QS21), and V4.34 (QS21)), and anti-N2 IgG antibodies were detected in the serums of ten of the subjects, among the twelve vaccinated. No antibody of type anti-N3 IgG was detected. The titration in anti-N2 antibody was negative in the serums of individuals V4.17 and V4.18. The antibody titration was three to five times greater than that of the negative control in the serums of V4.15, V4.16, V4.1 (QS21), V4.5 (QS21) and V4.21 (QS21). The antibody titration was five to ten times greater than that of the negative control in the serums of V4.6, V4.19 (QS21), and V4.28. Finally, the serums of patients V4.32 (QS21) and V4.34 (QS21) showed antibody titration at least 10 times greater than that of the negative control.

After 3 injections, no anti-G1 IgG antibody was detected, but anti-G2 IgG antibodies were detected in the serums of the 12 individuals vaccinated. The anti-G2 antibody titration was 2 to 3 times greater than that of the negative control for patient V4.18 (QS21), the antibody titration was 5 to 10 times greater than that of the negative control for individuals V4.16, V4.17, V4.5 (QS21), V4.19 (QS21) and V4.21 (QS21). The serums of patients V4.6, V4.15, V4.28, V4.1 (QS21), V4.32 (QS21) and V4.34 (QS21) had an antibody titration more than ten times greater than that of the negative control. The serums of 6 of the 12 individuals tested, V4.28, V4.1 (QS21), V4.5 (QS21), V4.19 (QS21), V4.32 (QS21) and V4.34 (QS21) contained specific anti-E antibodies.

Specific helper T cell response of the HIV-1 virus peptides.

The proliferative responses with respect to the soluble peptides obtained with the PBMC cells of the different individuals vaccinated are shown in table 15.

The NEF, GAG and ENV peptides caused proliferation of the donor PBMC only, after the vaccination. The PBMC of the individuals immunized with the lipopeptides (with or without QS21 adjuvant) proliferated against at least one peptide after 20 weeks (4 weeks after the third injection, for 8 subjects out of 10 given in table 15).

No proliferation was observed for the PBMC of individuals V4.15 and V4.17.

The PBMC of individual V4.6 proliferated in response to peptides N3, G1 and G2 with a proliferation index of between 4 and 10. An induction of the proliferation in response to G1, G2 and E was observed with the PBMC of individual V4.16. The PBMC of individual V4.28 were able to proliferate in response to peptides N1, N3, G2 and E. A proliferative response against peptides N1, G2 and E was observed for the PBMC of vaccinated individual V4.5.

A strong proliferation was observed in response to peptides N1, G2 and E with the PBMC of individual V4.19 (QS21), which in addition were able to proliferate in the presence of N2 and N3.

The PBMC of individual V4.21 proliferated in the presence of N1 and G2, while those of individuals V4.32 proliferated only in the presence of G2.

A proliferative response was observed in the presence of N1, N3, G2 and E with the PBMC of individual V4.34 (QS21).

Overall the third immunization with the lipopeptides induced a proliferative response against peptide N1 for five of the ten subjects treated, against N2 for one of the ten subjects treated, N3 for four of the ten subjects treated, G1 for two of the ten subjects treated, G2 for eight of the ten subjects treated, and finally E for five of the ten subjects treated.

The removal experiments performed with the PBMC from the different individuals vaccinated showed that the proliferation of the PBMC recovered after twenty weeks occurred preferentially via the helper $CD4^+$ T cells.

Induction of CTL activity specific to HIV

The PBMC obtained before and after the immunizations were stimulated in vitro and tested for their CTL activity specific to HIV.

The results of representative experiments are given in table 16.

The specific CTL activity was tested against the EBV autologous cell line, incubated with or without the NEF, GAG and ENV peptides. No anti-HIV response was detected with the PBMC recovered before the immunization. A specific CTL activity was detected in the PBMC collected three weeks after the immunization for nine of the twelve individuals.

Table 16 summarizes the cytotoxic activity of eight of the vaccinated individuals, the activity of one other individual being shown in figure 12.

At least one peptide contained in the lipopeptide vaccine induced specific CTL effector cells recognizing the HIV peptides.

For example, the PBMC of individual V4.6 recognized in a cytotoxic test the EBV autologous cells stimulated with peptides G2 and E. The PBMC of individual V4.16 recognized peptide N3 and E. The percentage of lysis was variable, weak for individual V4.16 recognizing peptide N3, intermediate for individual V4.18 with peptide N1 and strong for individual V4.5 (QS21) with peptides N2 and G2.

A specific CTL activity was also generated against peptides containing a minimal $CD8^+$ HIV antigenic determinant (individuals V4.16 and V4.28).

In order to evaluate whether the effector cells are $CD8^+$ T cells, as might be expected for the CTL specific for class-I restricted antigens, the $CD8^+$ or $CD4^+$ T lymphocytes were removed from the PBC and a cytotoxicity test was performed.

In representative experiments performed with the PBMC of individual V4.1 (figure 12), an effective lysis was observed of the autologous EBV cells incubated with the HIV peptides, for the PBMC and the $CD8^+$ cells. Enrichment

in $CD8^+$ cells increased the percentage of specific lysis. These results confirm that the anti-HIV cytotoxic activity operates via $CD8^+$ T cells.

It was also important to determine whether the cytotoxic T cells (CTL) recognized and lysed cells infected with the virus.

PBMC from individual V4.5, collected 20 weeks after immunization, and stimulated in vitro with peptide N2, were thus tested for their CTL activity against autologous targets infected with different viruses expressing the recombinant NEF protein. The results of representative experiments are given in figure 13. Anti-peptide N2 CTL obtained from individual V4.5 (QS21) recognized an antigen naturally modified by autologous EBV-LCL infected with recombinant viruses of the vaccine coding for the HIV-NEF genes obtained from different strains of HIV.

For the same effector/target ratio, CTL specific to the HIV virus recognized NEF-LAI and NEF-MN with the same effectiveness. A lower percentage of specific lysis was obtained for the NEF-A protein or the NEF-ROD protein.

These results show that the CTL obtained after vaccination with the lipopeptides are able to recognize different strains of the HIV virus.

<u>$CD8^+$ T cells secreting γ-interferon ex-vivo, specific to the HIV virus</u>

Effector $CD8^+$ T cells may exercise a lytic activity and/or produce lymphokines. The quantity of $CD8^+$ T cells secreting γ-interferon was thus evaluated by a specific ELISPOT test.

A recent study has shown that the intracellular inactivation of the hepatitis B virus occurs via $CD8^+$ T cells secreting specific and cytotoxic γ-interferon, which induced a protective immunity. This approach was used to identify the minimal antigenic determinants of $CD8^+$ T cells by sensitization of PBMC with the short peptides. All the short peptides used have already been described as being CTL antigenic determinants (see above).

An ELISPOT has also been used to quantify ex vivo the number of $CD8^+$ T cells secreting γ-interferon specific to HIV peptides in the PBMC of the vaccinated subjects (table 17).

Conclusion

This study has shown that a lipopeptide vaccine in the form of micelles, and without adjuvant, containing different HIV antigenic determinants contained in the viral proteins NEF, GAG and ENV of the HIV virus, is able to induce a strong and persistent multi-antigenic determinant B and T response in man.

TABLE 1: Antigenic determinants of BCR-ABL

Peptide		Sequence	Fixation to HLA
247-255		EDAELNPRF	B44
488-496		SELDLEKGL	B44
768-776		DELEAVPNI	B44
901-934	b2a2	KEDALQRPV	B44
902-935	b2a2	EDALQRPVA	B44
986-994		GEKLRVLGY	B44
1176-1184		EDTMEVEEF	B44
1252-1260		MEYLEKKNF	B44
1691-1699		NEEAADEVF	B44
49-57		VNQERFRMI	B 8
580-588		LFQKLASQL	B 8
722-730		ARKLRHVFL	B 8
786-794		ALKIKISQI	B 8
886-893		CVKLQTVH	B 8
928-936	b3a2	KALQRPVAS	B 8
1830-1838		GAKTKATSL	B 8
1975-1983		IQQMRNKFA	B 8
1977-1984		QMRNKFAF	B 8
252-260		NPRFLKDNL	B7
329-338		TPDCSSNENL	B7
693-701		TPRRQSMTV	B7
1058-1066		SPGQRSISL	B7
1196-1205		HPNLVQLLGV	B7
1560-1569		SPKPSNGAGV	B7
1717-1725		KPLRRQVTV	B7
1878-1884		SPAPVPSTL	B7
36-44		ERCKASIRR	B27
71-79		DRQRWGFFRR	B27
575-583		QRVGDLFQK	B27

TABLE 1: Antigenic determinants OF BCR-ABL

Peptide		Sequence	Fixation to HLA
834-842		FRVHSRNGK	B27
642-650		LLYKPVDRV	A2
684-692		FLSSINEEI	A2
708-716		QLLKDSFMV	A2
714-722		FMVELVEGA	A2
817-825		KLSEQESLL	A2
881-889		MLTNSCVKL	A2
908-917		GLYGFLNVIV	A2
912-920		FLNVTVHSA	A2
1240-1248		VLLYMATQI	A2
1903-1911		FIRLISTRV	A2
1932-1940		VVLDSTEAL	A2
50-58		NQERFRMIY	A1
223-231		VGDASRPPY	A1
549-558		KVPELYEIHK	A3/A11
583-591		KLASQLGVY	A3/A11
715-724		MVELVEGARK	A3/A11
916-923		IVHSATGFK	A3/A11
920-928	b3a2	ATGFKQSSK	A3/A11
924-932	b3a2	KQSSKALQR	A3/A11
1156-1165		EVYEGVWKKY	A3/A11
1311-1320		SLAYNKFSIK	A3/A11
1499-1509		NLFSALIKK	A3/A11
1724-1734		TVAPASGLPHK	A3/A11
1905-1914		LISTRVSLRK	A3/A11
1922-1930		RIASGAITK	A3/A11
924-936 b3a2		KQSSKALQRPVAS	DR4

TABLE 2 - Antigenic determinants of p53

- antigenic determinants of p53 binding to HLA-A1:
RVEGNLARVEY (196-205)
GSDCTTIHY (226-234)
- antigenic determinants of p53 binding to HLA-A2:
LLPENNVLSPL (25-35)
RMPEAAPPV (65-73)
RMPEAAPRV
ALNKMFCQL (129-137)
STPPPGTRV (149-157)
GLAPPQHLIRV (187-197)
LLGRNSFEV (264-272)
PLDGEYFTL (322-330)
- antigenic determinants of p53 binding to HLA-A3:
RVRAMAIYK (156-164)
RRTEEENLR (282-290)
ELPPGSTKR (298-306)
- antigenic determinants of p53 binding to HLA-B7:
LPENNVLSPL (26-35)
APRMPEAAPPV (63-73)
APRMPEAAPRV
APPQHLIRV (189-197)
RPILTIITL (249-257)
KPLDGETYFTL (321-330)
- antigenic determinants of p53 binding to HLA-B8:
CQLAKTCPV (135-143)
GLAPPQHLI (187-195)
NTFRHSVVV (210-218)
- antigenic determinants of p53 binding to HLA-B51:
LLPENNVLSPL (25-35)
RMPEAAPPV (65-73)
LIRVEGNLRV (194-203)

TABLE 3

Antigenic determinants of proteins E_6 and E_7

YMLDLQPETT (E7 11-20)
LLMGTLGIV (E7 82-90)
TLGIVCPI (E7 86-93)
TIHDIILECV (E6 29-38)
KLPQLCTEL (E6 18-26)
RPPKLPQL (E6 8-15)
LRREVYDFAFRDLCIVYRDGNPY (E6 45-67)
ISEYRHYCY (E6 80-88)
EKQRHLDKKQRFHNIRGRWT (E6 121-140)
GQAEPDRAHYNIVTF (E7 43-57)
QAEPDRAHY (E7 44-52)
EPDRAHYNIV (E7 46-55)

TABLE 4: Antigenic determinants of the HIV-1 virus

HLA-A1

(Nef 96-106: GLEGLIHSQRR
(Nef 121-128: FPDWQNYT
(Nef 137-145: LTFGWCYKL
(Nef 184-191: RFDSRLAF
(Nef 195-202: ARELHPEY

HLA-A2

Gpl20 121-129: KLTPLCVTL
P17 77-85: SLYNTVATL
RT 200-208: ALVEICTEM
RT 275-285: VLDVGDAYFSV
RT 346-354: KIYQYMDDL
RT 368-376: KIEELRQHL
RT 376-387: LLRWGLTTPDK
RT 476-484: ILKEPVHGV
RT 588-596: PLVKLWYQL
RT 683-692: ELVNQIIEQL
Nef 136-145: PLTFGWCFKL
Nef 180-189: VLQWRFDSRL
Nef 190-198: ALHHVAREL
Gp41 818-826: SLLNATVDI
P24 185-193: DLNTMLNTV
RT 346-354: VIYQYMDDL
RT 588-596: PLVKLWYQL
Pro 143-152: VLVGPTPVNI
(Gp120 37-44: TVYYGVPV
(Gp120 115-122:SLKPCVKL
(Gp120 313-321: RIQRGPGRA
(Gp120 197-205: TLTSCNTSV
(Gp120 428-435: FINMWQEV
(Gp 41 836-844: VVQGAYRAI
(p24 219-228: HAGPIAPGQM
(p15 422-431: QMKDCTERQA
(p15 448-456: FLQSRPETA
(RT 681-691: ESELVNQIIEG

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-A3

P17 18-26: KIRLRPGGK
P17 20-28: RLRPGGKKK
RT 200-210: ALVEICTEMEK
RT 325-333: AIFQSSMTK
RT 359-368: DLEIGQHRTK
Nef 73-82: QVPLRPMTYK
Gp120 37-46: TVYYGVPVWK
Gp41 775-785: RLRDLLLIVTR
P17 18-26: KIRLRPGGK

HLA-A11

RT 325-333: AIFQSSMTK
RT 507-517: QIYQEPFKNLK
Nef 73-82: QVPLRPMTYK
Nef 84-92: AVDLSHFLK
p24 349-359: ACQVGGPGHK
P17 83-91: ATLYCVHQR

HLA-A24 (A9)

Gp120 52-61: LFCASDAKAY
Gp41 591-598: YLKDQQLL
or 590-597 RYLKDQQLL
(RT 484-492: VYYDPSKDL
(RT 508-516: IYQEPFKNL
(RT 681-691: ESELVNQIIEG

HLA-A25 (A10)

P24 203-212: ETINEEAAEW

HLA-A26 (A10)

P24 167-175: EVIPMFSAL

HLA-A30 (A19)

(Gp41 845-852: RAIRHIPRR

HLA-A31 (A19)

(Gp41 775-785: RLRDLLLIVTR

HLA-A32 (A19)

Gp120 424-432: RIKQIINMW
(Gp41 774-785: HRLRDLLLI
RT 559-568: PIQKETWETW

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-A33 (A19)

(P24 266-275 :IILGLNKIVR

HLA-B7

RT 699-707: YLAWVPAHK
Nef 68-77: FPVTQVPLR
Nef 128-137: TPGPGVRYPL
Gp120 303-312: RPNNNTRKSI
Gp41 848-856: IPRRIRQGL
RT 699-707: YLAWVPAHK

HLA-B8

Gp120 2-10: RVKEKYQHL
P17 24-32 :GGKKKYKLK
Nef 90-97: FLKEKGGL
P24 259-267: GEIYKRWII
Gp41 591-598: YLKDQQLL
(Gp41 849-856: PRRIRQGL
or 851-859: RIRQGLERIL
(P24 329-337: DCKTILKAL
(RT 185-193: GPKVKQWPL
(Nef 182-189: EWRFDSRL

HLA-B14

Gp41 589-597: ERYLKDQQL
P24 298-306: DRFYKTLRA
(P24 183-191 ?: DLNTMLNTV
(p24 304-313: LRAEQASVQEV
(p24 305-313: RAEQASVQEV

HLA-B18

(Nef 135-143: YPLTFGWCY
(Nef 135-143: YPLTFGWCF

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-B27

P24 263-272: KRWIILGLNK
Nef 73-82: QVPLRPMTYK
Nef 134-141: RYPLTFGW
or 133-141 : YPLTFGW
Gp41 589-597 ERYLKDQQL
(Gp41 791-800: GRRGWEALKY

HLA-B35

Gp120 78-86: DPNPQEVVL
Gp120 257-265: RPVVSTQLL
RT 285-294: VPLDKDFRKY
RT 323-331: SPAIFQSSM
RT 342-350: NPDIVTYQY (consensus clade B)
RT 460-468: IPLTEEAEL
RT 598-608: EPIVGAETFY
Nef 68-76: FPVRPQVPL
Nef 74-81: VPLRPMTY
Gp41 611-619: TAVPWNASW
Gp120 42-52: VPVWKEATTTL
P17 124-132: NSSQVSQNY (consensus clade B)
P24 254-262: PPIPVGEIY(consensus clade B)

HLA-B37

Nef 120-128: YFPDWQNYT

HLA-B44 (B12)

P24 178-186: SEGATPQDL
(p24 175-184: LESGATPQDL

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-B51 (B5)

gp41 562-570: RAIEAQQHL
RT 200-208: ALVEICTEM
RT 209-217: EKEGKISKI
RT 295-302: TAFTIPSI

HLA-B52 (B5)

Nef 190-198: AFHHVAREL

HLA-B55 (B22)

Gp120 42-51: VPVWKEATTTL

HLA-B57 and B58 (B17)

P24 240-249: TSLTQEQIGW
Nef 116-125: HTQGYFPDWQ
or 116-124: HTQGYFPDW
Nef 120-128: YFPDWQN
(P24 147-155: ISPRTLNAW
(P24 164-172: FSPEVIPMF

HLA-Bw62 (B15)

P17 20-29: RLRPGGKKKY
P24 268-277: LGLNKIVRMY
RT 427-438: LVGKLNWASQIY
Nef 84-91: AVDLSHFL
Nef 117-127: TQGYFPDWQNY

HLA-Cw4

gp120 380-388: SFNCGGEFF

HLA-Cw8

RT 663-672: VTDSQYALGI
P24 305-313: RAEQASQEV
Nef 82-91: KAALDLSHPL

HLA-Cw?

P24 308-316: QATQEVKNW

TABLE 5 - Antigenic determinants of human melanoma

Gene/protein	MHC restriction	Peptide	Amino acid positions
Tyrosinase	HLA-A2	MLLAVLYCL	1-9
	HLA-A2	YMNGTMSQV	369-377
		YMDGTMSQV	
	HLA-A24	AFLPWHRLF	206-214
	HLA-B44	SEIWRDIDF	192-200
	HLA-DR4	QNILLSNAPLGPQFP	56-70
		SYLQDSDPDSFQD	450-462
Pmel17^{gp100}	HLA-A2	KTWGQYWQV	154-162
	HLA-A2	AMLGTHTMEV	177-186
	HLA-A2	MLGTHTMEV	178-186
	HLA-A2	ITDQVPFSV	209-217
	HLA-A2	YLEPGPVTA	280-288
	HLA-A2	LLDGTATLRL	457-466
	HLA-A2	VLYRYGSFSV	476-485
	HLA-A2	SLADTNSLAV	570-579
	HLA-A3	ALLAVGATK	17-25
Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26(7)-35
	HLA-A2	ILTVILGVL	32-40
GP$^{75TRP-1}$	HLA-A31	MSLQRQFLR	
TRP-2	HLA-A31	LLGPGRPYR	197-205

TABLE 6: Tumour Antigenic determinants resulting from de mutations

Gene/protein	Tumour	MHC restriction	Peptide	Amino acid positions
MUM-1	Melanoma	HLA-B44	EEKLIVVLF	30-38
CDK4	Melanoma	HLA-A2	ACDPHSGHFV	23-32
β-catenine	Melanoma	HLA-A24	SYLDSGIHF	29-37
HLA-A2	Renal carcinoma			-
CASP-8	Squamous carcinoma of head and neck	HLA-B35	FPSDSWCYF	476-484

TABLE 7

Antigens common to different tumours

Gene	Normal expression tissue	MHC restriction	Antigenic peptide	Amino acid positions
MAGE-1	testicles	HLA-A1 HLA-Cw16	EADPTGHSY SAYGEPRKL	161-169 230-238
MAGE-3	testicles	HLA-A1 HLA-A2 HLA-B44	EVDPIGHLY FLWGPRALV MEVDPIGHLY	168-176 271-279 167-176
BAGE	testicles	HLA-Cw16	AARAVFLAL	2-10
GAGE-1/2	testicles	HLA-Cw6	YRPRPRRY	9-16
RAGE-1	retina	HLA-B7	SPSSNRIRNT	11-20
GnTV	none	HLA-A2	VLPDVFIRC	38-64
mucin	breasts during lactation	no restriction	PDTRPAPGSTAPPA HGVTSA*	

* Aberrant transcript of the N-acetyl glucosaminyl transferase V (GnTV) found only in melanomas

TABLE 8

LIPOPEPTIDES	FILTRATION YIELD
NEF 66	quantitative
NEF 117	80%
NEF 182	quantitative
GAG 183	80%
GAG 253	77%
ENV	quantitative

TABLE 9

Peptide	Solvent	Concentration (mg/ml)	Volume removed (ml)	Filtration yield (%) after mixing
NEF 66	water	5	1	95
NEF 117	AcOH 25%	5	1	81
NEF 182	AcOH 25%	5	1	92
GAG 183	AcOH 80%	10	0.5	73
GAG 253	AcOH 25%	5	1	31
ENV	water	5	1	95

TABLE 10

Peptide	Solvent	Concentration (mg/ml)	Volume removed (ml)	Filtration yield (%) after mixing*
NEF 66	AcOH 80%	20	0.250	quantitative
NEF 117	AcOH 80%	20	0.250	quantitative
NEF 182	AcOH 80%	20	0.250	quantitative
GAG 183	AcOH 80%	20	0.250	quantitative
GAG 253	AcOH 80%	20	0.250	quantitative
ENV	AcOH 80%	20	0.250	quantitative

* to within the precision of the determination

TABLE 11

Peptide	Exact weight*** (mg)	Peptide net	Quantity expected* (µg per dose)	Quantity obtained*** (µg per dose)	yield** (%)
NEF 66	764	641	550	505 ± 15	89.14 - 94.6
NEF 117	739	641	550	621 ± 21	109.08 - 116.72
NEF 182	742	641	550	545 ± 16	96.23 - 102.05
GAG 183	741	642	550	478 ± 13	84.50 - 89.23
GAG 253	780	641	550	571 ± 28	98.76 - 108.95
ENV	810	642	550	593 ± 17	104.71 - 110.89

* the target dose was 500 µg per peptide: an overdose of 10% was deliberately included at the time of weighing, given the yields obtained during the preparation of batch CK6

** the yield ranges reflect the precision of the determination, and not a significant variation from one flask to another

*** the values in excess are due to imprecisions in the weighings of electrostatic powders by an operator wearing a standard pressure suit

TABLE 12 - Test of uniformity of concentration

	nef 66	nef 117	nef 182	gag 183	gag 253	env 303
	14.40	17.74	16.19	13.28	16.74	17.53
	14.38	17.75	16.21	13.27	17.27	17.63
	14.67	16.36	16.61	13.41	16.89	18.36
sample 1	14.49	17.28	16.34	13.32	16.97	17.84
	13.42	17.11	15.32	12.66	15.56	16.36
	13.81	17.04	15.32	12.67	15.89	16.51
	13.77	17.06	15.33	12.45	15.16	16.41
sample 2	13.67	17.07	15.32	12.59	15.54	16.43
	13.58	17.08	15.33	12.68	15.64	16.29
	13.70	17.08	15.31	12.62	15.82	16.28
	13.59	17.05	15.31	12.32	14.85	16.37
sample 3	13.62	17.07	15.32	12.54	15.44	16.31
	13.20	16.80	15.14	12.23	16.05	16.15
	14.53	17.34	15.74	13.06	15.93	17.17
	13.49	16.86	15.17	12.31	15.44	16.15
sample 4	13.74	17.00	15.35	12.53	15.80	16.49
	13.88	17.21	15.40	12.52	14.78	16.80
	13.94	17.17	15.39	12.59	15.20	16.72
	13.98	17.19	15.47	12.96	15.49	16.71
sample 5	13.94	17.19	15.42	12.69	15.16	16.74
	14.03	17.26	15.75	11.62	15.78	16.97
	13.99	17.20	15.73	11.39	15.77	17.02
	14.20	17.26	15.74	12.19	15.90	16.80

TABLE 12 - Test of uniformity of concentration (continued)

sample 6	14.07	17.24	15.74	11.73	15.81	16.93
	13.78	17.29	15.67	12.69	16.13	18.04
	13.94	17.22	15.57	12.67	16.40	17.50
	13.95	17.23	15.55	12.28	16.19	17.37
sample 7	13.89	17.25	15.60	12.55	16.24	17.64
	13.84	17.06	15.38	12.50	15.62	17.90
	13.65	17.09	15.45	12.44	16.02	17.73
	13.73	16.94	15.37	nd	16.21	17.54
sample 8	13.74	17.03	15.40	12.47	15.95	17.73
	14.03	17.40	15.66	12.77	16.46	18.56
	14.07	17.33	15.72	11.92	16.61	18.41
	13.89	17.39	15.72	12.68	15.94	18.37
sample 9	14.00	17.37	15.70	12.46	16.34	18.45
	13.34	16.88	15.33	12.07	14.70	17.92
	13.71	17.24	15.66	12.36	15.12	18.50
	13.53	16.93	15.44	12.28	14.44	18.01
sample 10	13.53	17.02	15.48	12.24	14.76	18.14
	13.72	17.22	15.64	12.41	14.89	18.32
	13.75	17.33	15.72	12.36	14.82	18.31
	13.67	17.21	15.72	11.86	14.61	18.69
sample 11	13.71	17.25	15.69	12.21	14.77	18.44
	13.62	17.11	15.60	12.28	14.75	18.42
	13.74	17.13	15.70	12.44	14.98	18.31
	13.75	17.16	15.63	12.51	15.37	18.32
sample 12	13.70	1 7. 13	15.65	12.41	15.03	18.35
	13.32	16.36	14.74	12.47	16.17	15.44
	13.31	16.41	14.68	12.51	16.26	15.56
	13.34	16.38	14.67	12.53	16.17	15.43

TABLE 12 - Test of uniformity of concentration (continued)

sample 13	13.32	16.38	14.70	12.50	16.20	15.48
	13.76	16.72	14.75	12.67	16.10	15.59
	13.56	16.35	14.75	12.64	16.16	15.64
	13.60	16.67	14.76	12.64	16.06	15.64
sample 14	13.64	16.58	14.76	12.65	16.10	15.62
	13.36	16.40	14.53	12.48	15.90	15.64
	13.41	16.35	14.57	12.52	15.84	15.80
	13.44	16.43	14.50	12.46	15.77	15.60
sample 15	13.40	16.39	14.53	12.49	15.84	15.68

m	13.69	16.91	15.28	12.49	15.81	16.82
standard deviation	0.27	0.37	0.42	0.28	0.59	1.19
t's	0.58	0.79	0.89	0.59	1.24	2.51
min	13.11	16.12	14.39	11.91	14.57	14.30
max	14.27	17.70	16.18	13.08	17.05	19.33

t = 2.110 3 deviant values on sample 1 (probably dilution error)

Test of uniformity of concentration

Min accepted (-15%)	11.64	14.37	12.99	10.62	13.44	14.29
Max accepted (+15%)	15.74	19.45	17.58	14.37	18.18	19.34
Observed deviation	4.23%	4.67%	5.84%	4.70%	7.84%	14.95%

TABLE 13

anti-7 peptide CTL lines	Peptides recognized	Short peptides recognized
92102	GAG 246-281	
92105	NEF 125-147	
92109	NEF 101-126	NEF 101-110 NEF 116-126
	NEF 125-147	NEF 128-136
	NEF 155-178	NEF 169-178
	NEF 201-225	NEF 215-225
	GAG 246-281	
92120	GAG 246-281	
92125	NEF 155-178	NEF 169-178
92129	NEF 125-147 NEF 155-178 NEF 201-225	NEF 128-136 NEF 169-178 NEF 201-211 NEF 211-219
92117	negative	
92127	NEF 101-126 NEF 125-147 NEF 155-178	

TABLE 14
Detection of specific antibodies of peptides of proteins NEF, GAG and ENV of the HIV virus, in the serum of volunteers immunized with a mixture of six lipopeptides

Volunteer[a]	Recovery period	Peptide recognized					
		N1	N2	N3	G1	G2	E
V4.6	W20	2.1	7.2	1.0	1.0	10.2	1.2
V4.15	W20	1.3	4.8	1.2	1.3	11.2	1.5
V4.16	W20	1.2	4.7	1.2	1.1	9.7	1.3
V4.17	W20	1.7	1.8	1.0	1.1	8.0	1.2
V4.18	W20	1.1	1.2	1.0	1.3	2.2	1.1
V4.28	W20	7.8	8.8	1.5	1	15	5.7
V4.1 (QS21)	W20	3.1	3.2	1.1	1.2	11.5	4.7
V4.5(QS21)	W20	1.2	4.2	1.3	1.1	8.1	2.1
V4.19 (QS21)	W20	1.2	5.3	1.2	1.3	5.1	3.8
V4.21 (QS21)	W20	1.2	4.7	1.0	1.2	9.3	1.9
V4.32 (QS21)	W20	6.6	14	1.8	1.9	21	4
V4.34 (QS21)	W20	7.1	21.2	1.2	1.8	36	8

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21)
[b] The serums of the volunteers were recovered before injection of the lipopeptides, and twenty weeks after. The three injections of the six lipopeptides were administered at 0.4 and 16 weeks.
[c] The detection of the specific antibodies of the peptides of the HIV virus was performed using an ELISA assay with serum dilution to 1/100. The ELISA assay plates were covered with NEF 66-97 (N1), NEF 117-147 (N2), NEF 182-205 (N3), GAG 183-214 (G1), GAG 253-284 (G2) or V3 ENV 303-335 (E).

TABLE 15
Proliferative responses of the PBMC of the volunteers with lipopeptides NEF, GAG and ENV

Volunteer[a]	Recovery period	Proliferation index[c]			
		N1	N2	N3	G1
V4.6	W0	1.3	1.0	1.0	1.3
	W20	2.4	3.1(±1)	10(±7)	4.5(±1.1)
V4.15	W0	1.0	1.0	1.3	1.0
	W20	1.9	2.2	1.6	1.2
V4.16	W0	1.0	1.2	1.3	1.3
	W20	1.1	1.1	2.2	4.6(±0.6)
V4.17	W0	2.5	1.3	1.4	1.9
	W20	1.5	1.1	1.6	2.0
V4.18	W0	1.0	1.3	1.5	1.8
	W20	nd	nd	nd	nd
V4.28	W0	1.3	2.2	1.7	1.2
	W20	3.8(±0.6)	1.2	21(±2)	1.2

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

[c] $2\ 10^5$ cells were cultivated with 1 μg/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

[d] The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1 μg/ml of PHA, PPD and SEB.

TABLE 15 continued (1)
Proliferative responses of the PBMC of the volunteers
with lipopeptides NEF, GAG and ENV

Volunteer[a]	Recovery period	Proliferation index[c]		
		G2	E	Proliferation induced by the culture medium[d]
V4.6	W0	1.0	1	871 (±25)
	W20	7.2(±0.7)	3.6(±0.9)	280 (±32)
V4.15	W0	1.4	1.5	1657 (±182)
	W20	1.6	2.1	252 (±30)
V4.16	W0	1.8	1.5	3830 (±232)
	W20	3.6(±0.5)	3.9(±0.7)	1000 (±168)
V4.17	W0	2.0	2.3	5708 (±470)
	W20	2.0	2.8	1228 (±54)
V4.18	W0	3.3(±0.7)	1.3	460 (±49)
	W20	nd	nd	nd
V4.28	W0	1.2	1.2	869 (±36)
	W20	8.2(±1.6)	7.6(±2.2)	2558 (±186)

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

[c] $2\ 10^5$ cells were cultivated with 1 µg/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

[d] The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1 µg/ml of PHA, PPD and SEB.

TABLE 15 continued (2)
Proliferative responses of the PBMC of the volunteers
with lipopeptides NEF, GAG and ENV

Volunteer[a]	Recovery period	Proliferation index[c]			
		N1	N2	N3	G1
V4.1 (QS21)	W0	1.0	1.1	1.0	1.1
	W20	nd	nd	nd	nd
V4.5(QS21)	W0	1.5	1.6	1.5	1.6
	W20	4.6(±1.2)	3.1(±0.3)	1.5	2.0
V4.19 (QS21)	W0	1.0	1.1	1.2	1.1
	W20	24.3(±3.1)	8.5(±5)	4.4(±1.2)	3.1(±2.5)
V4.21 (QS21)	W0	1.3	1.3	1.2	3.9(±1)
	W20	6.5(±3)	1.4	2.3	2.8
V4.32 (QS21)	W0	1.0	1.0	1.1	1.3
	W20	1.0	1.0	1.7	1.2
V4.34 (QS21)	W0	1.0	1.1	1.1	1.2
	W20	3.4(±0.2)	1.1	3.3(±0.1)	2.2

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

[c] $2\ 10^5$ cells were cultivated with 1 μg/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

[d] The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1 μg/ml of PHA, PPD and SEB.

TABLE 15 continued (3)
Proliferative responses of the PBMC of the volunteers
with lipopeptides NEF, GAG and ENV

Volunteer[a]	Recovery period	Proliferation index[c]		
		G2	E	Proliferation induced by the culture medium[d]
V4.1 (QS21)	W0	1.0	1.0	3107 (±521)
	W20	nd	nd	nd
V4.5(QS21)	W0	1.5	1.3	341 (±20)
	W20	3.9(±0.3)	5.0(±2.2)	776 (±60)
V4.19 (QS21)	W0	1.0	1.0	918 (±102)
	W20	11.0(±2.7)	9.4(±2.8)	497 (±168)
V4.21 (QS21)	W0	1.3	1.4	322 (±21)
	W20	11.3(±4)	3.3(±1.9)	1052 (±82)
V4.32 (QS21)	W0	1.2	1.2	4448 (±75)
	W20	10.1(±1.5)	0.9	245 (±30)
V4.34 (QS21)	W0	1.2	1.2	5383 (±309)
	W20	4.4(±0.6)	3.1(±0.1)	7381 (±280)

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

[c] $2\ 10^5$ cells were cultivated with 1 µg/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

[d] The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1 µg/ml of PHA, PPD and SEB.

TABLE 16
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

Lipopeptide incubated with target cells	V4.6[a]		V4.16		V4.18		V4.28	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	70/1	70/1	50/1	50/1	80/1	80/1	10/1	10/1
None	5%	11%	2%	12%	8%	14%	5%	8%
NEF 66-97	9%	17%	8%	18%	11%	40%	12%	19%
NEF 117-147	9%	16%	2%	13%	6%	6%	19%	12%
NEF 182-205	4%	15%	2%	24%	6%	6%	2%	4%
E/T Ratio	70/1	70/1	30/1	30/1	55/1	55/1	10/1	10/1
None	4%	18%	5%	5%	8%	6%	2%	2%
GAG 183-214	7%	14%	9%	10%	nd	nd	2%	2%
GAG 253-284	9%	49%	11%	20%	6%	26%	2%	2%

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).
[b] The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with ^{51}Cr.
[c] The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).
[d] The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10^3 marked target cells, incubated with varying quantities of effector cells.

TABLE 16 (continued 1)
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

Lipopeptide incubated with target cells	V4.5 (QS21)		V4.19 (QS21)		V4.21 (QS21)		V4.34 QS21)	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	100/1	100/1	60/1	60/1	40/1	40/1	35/1	35/1
None	16%	31%	2%	6%	2%	2%	10%	2%
NEF 66-97	10%	23%	0%	15%	2%	2%	2%	2%
NEF 117-147	18%	47%	2%	27%	2%	2%	2%	2%
NEF 182-205	10%	31%	0%	0%	2%	2%	2%	2%
E/T Ratio	140/1	140/1	60/1	60/1	46/1	46/1	35/1	35/1
None	23%	11%	0%	0%	2%	2%	2%	2%
GAG 183-214	21%	11%	0%	0%	2%	2%	2%	23%
GAG 253-284	20%	68%	0%	9%	2%	2%	5%	5%

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with ^{51}Cr.

[c] The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

[d] The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10^3 marked target cells, incubated with varying quantities of effector cells.

TABLE 16 (continued 2)
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

Lipopeptide incubated with target cells	V4.6[a]		V4.16		V4.18		V4.28	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	70/1	70/1	25/1	25/1			10/1	10/1
None	3%	6%	32%	23%	nd	nd	2%	2%
V3 ENV 303-335	2%	36%	12%	49%	nd	nd	2%	17%
E/T Ratio			50/1	50/1			10/1	10/1
None			13%	23%			2%	2%
anti-A1			2%	48%			5%	34%
anti-A3			nd	nd			nd	nd

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

[b] The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with ^{51}Cr.

[c] The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

[d] The E/T ratio (ratio effector cells/target cells) corresponds to 5×10^3 marked target cells, incubated with varying quantities of effector cells.

TABLE 16 (continued 3)
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

Lipopeptide incubated with target cells	V4.5 (QS21)		V4.19 (QS21)		V4.21 (QS21)		V4.34 QS21)	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	60/1	60/1	60/1	60/1	86/1	86/1	35/1	35/1
None	22%	14%	0%	2%	7%	13%	5%	3%
V3 ENV 303-335	24%	16%	2%	6%	2%	23%	3%	3%
E/T Ratio							35/1	35/1
None							2%	2%
anti-A1							nd	nd
anti-A3							2%	2%

[a] The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).
[b] The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with ^{51}Cr.
[c] The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).
[d] The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10^3 marked target cells, incubated with varying quantities of effector cells.

TABLE 17

CD8+ T cells secreting γ-interferon/ex vivo assay

Number of cells secreting γ-interferon/1 x 10^6 cells

		V4.18 A2/11 B44/60		V4.5 (QS21) A2/11 B18/27		V4.21 (QS21) A1 B8	
		W0	W20	W0	W20	W0	W20
HLA-A1	NEF 121-128					1	41
	NEF 137-145					1	6
	NEF 184-191					6	26
	NEF 195-202					1	1
HLA-A2	NEF 136-145	0	0	0	4		
	NEF 190-198	nd	nd	4	16		
	GAG 183-191	nd	nd	2	16		
HLA-A11	NEF 73-82[c]	0	15	0	0		
	NEF 84-92	0	55	nd	nd		
	EBN 416-424	500	500	66	63		
HLA-B8	NEF 90-97					1	51
	NEF 182-189					1	26
HLA-B27	NEF 134-141			2	14		
	GAG 263-272			0	9		
HLA-B18	NEF 135-143			0	9		